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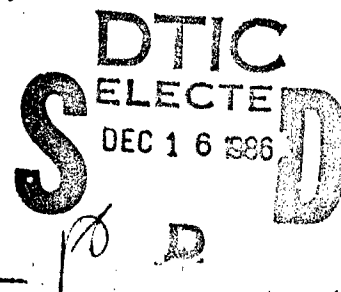
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STUDY OF THE PATHOGENICITY OF AEROMONAS HYDROPHILA FOR MAN

ANNUAL REPORT
September 1, 1984 - November 30, 1985

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NOVEMBER 1985



20030122017

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3024

University of Texas Health Science Center at Houston
Houston, Texas 77030

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE			Approved for public release; distribution unlimited.		
4. PERFORMING ORGANIZATION REPORT NUMBER(S)			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
6a. NAME OF PERFORMING ORGANIZATION University of Texas Health Science Center at Houston		6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION		
6c. ADDRESS (City, State, and ZIP Code) Houston, Texas 77030			7b. ADDRESS (City, State, and ZIP Code)		
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command		8b. OFFICE SYMBOL (If applicable) SGRD-RMI-S	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-83-C-3024		
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21701-5012			10. SOURCE OF FUNDING NUMBERS		
			PROGRAM ELEMENT NO. 63750A	PROJECT NO. 3M4- 63750D808	TASK NO. AA
			WORK UNIT ACCESSION NO 022		
11. TITLE (Include Security Classification) (U) Study of the Pathogenicity of <u>Aeromonas Hydrophila</u> for Man					
12. PERSONAL AUTHOR(S) DuPont, Herbert L., M.D.					
13a. TYPE OF REPORT Annual		13b. TIME COVERED FROM 9/1/84 TO 11/30/85		14. DATE OF REPORT (Year, Month, Day) 1985 November	
15. PAGE COUNT					
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP			
06	13		Aeromonas Hydrophila		
06	03				
19. ABSTRACT (Continue on reverse if necessary and identify by block number)					
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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input type="checkbox"/> UNCLASSIFIED/DUNLIMITED <input checked="" type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED		
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian			22b. TELEPHONE (Include Area Code) 301/663-7325		22c. OFFICE SYMBOL SGRD-RMI-S

19. Abstract - continued

In studies looking at the etiology of diarrhea among groups of U.S. adult students in Mexico we found that non-EPEC serotypes of enteroadherent *E. coli* (EAEC) were isolated from 12% of diarrhea cases overall and from 30% of the patients from whom no other agents were identified. Six of 13 students from whom paired sera were collected showed a fourfold or greater rise in antibodies in serum. Two strains of EAEC were selected for study in volunteers. In doses of 7×10^8 and 1×10^{10} viable cells, strain JM 221 produced a diarrheal illness in 4 of 8 subjects while 1 of 8 fed strain 189 developed a diarrheal illness. The test strains were recovered from stool throughout the study indicating intraluminal or intraintestinal replication of the test strains.

FOREWORD

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products of services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Summary

Five Aeromonas hydrophila strains were selected for study from among more than 50 candidate strains collected from Thailand, Western Australia, Canada, and the United States. They were selected for the study because of possession of well characterized virulence properties or because they were clearly implicated in cases of human diarrheal illness. All were felt to be pathogenic by laboratory assays. The strains were tested biochemically and toxin production and hemagglutination patterns were characterized. Each strain was then fed to groups of 3 or 4 adult volunteers. The strains were shed by the volunteers only sporadically and except for two volunteers transiently passing unformed stools, a sustained illness did not develop. This study failed to show a relationship between virulence properties as we now understand them and pathogenicity of Aeromonas for human subjects.

In studies looking at the etiology of diarrhea among groups of U.S. adult students in Mexico we found that non-EPEC serotypes of enteroadherent E. coli (EAEC) were isolated from 12% of diarrhea cases overall and from 30% of the patients from whom no other agents were identified. Six of 13 students from whom paired sera were collected showed a fourfold or greater rise in antibodies in serum. Two strains of EAEC were selected for study in volunteers. In doses of 7×10^8 and 1×10^{10} viable cells, strain JM 221 produced a diarrheal illness in 4 of 8 subjects while 1 of 8 fed strain 189 developed a diarrheal illness. The test strains were recovered from stool throughout the study indicating intraluminal or intraintestinal replication of the test strains.

TABLE OF CONTENTS

	Page
Nature of Work Being Reported	5
Statement of the Problem	5
Background	5
Approach to Problem	6
Results	8
Discussion and Conclusions	9
Literature Cited	16
Distribution List	19

Tables

Table 1. Source of <u>Aeromonas hydrophila</u>	11
Table 2. Biochemical Characterization and Antimicrobial Susceptibility of <u>A.</u> <u>hydrophila</u> Strains	12
Table 3. Toxins Produced and Hemagglutination Patterns of <u>A. hydrophila</u> Strains	13
Table 4. Administered Dose and Excretion of <u>A. hydrophila</u> in Volunteers	14
Table 5. Challenge Dose, Strain Excretion and Occurrence of Symptoms - Two Enteroadherent <u>E. coli</u> Strains	15

Nature of Work Being Reported

The annual report deals within the last 12 months of a total of 24 months concerning contract No. C-3024. The project has dealt with the establishment of the volunteer pool, the accumulation and evaluation of test strains and the administration of three dosages of five strains of A. hydrophila and two dosage levels of two nonenteropathogenic serotype but enteroadherent E. coli strains.

Statement of the Problem

Approximately 50% of acute diarrhea is associated with the passage of unformed stools which are negative for all known enteric pathogens. Studies carried out in many regions of the world have shown that antimicrobial agents can be used to successfully treat or prevent a portion of the illness offering indirect evidence that as yet undefined bacterial agents will prove to be important causes. Two leading bacterial candidates as potential causes of the undiagnosable illness are A. hydrophila and nonenteropathogenic but enteroadherent E. coli. A. hydrophila has recently been isolated in a high frequency of diarrhea cases which occur in Thailand, Western Australia, and Canada. The adherent E. coli have been isolated in 12% of cases of acute diarrhea cases occurring in U.S. adults studying in Mexico and may explain up to one-third of the previously undiagnosable illness. The present study was designed to establish the virulence of these strains for man and to characterize the pathogenesis of infection for those capable of producing illness. Additionally, this volunteer program remains one of the few facilities in the country where enteric infections can be induced in man for the purpose of studying experimental infection. Priority for future study will be given for enteropathogens of interest to the U.S. Army.

Background

Diarrheal diseases are a major cause of morbidity in all areas of the world and mortality in developing regions. A large proportion of acute diarrhea is undiagnosable etiologically, yet these cases often respond promptly to antimicrobial therapy (1-3). This finding suggests that undefined bacterial agents may be responsible for acute diarrhea.

Various strains of Aeromonas hydrophila possess characteristics associated with virulence of enteropathogenic bacteria. Most strains produce a heat-labile cytotoxin as well as enterotoxins (4-6). A recent report indicates that a cholera-like toxin may be produced as well by Aeromonas strains (Houston, ASM 1984). Other A. hydrophila are also capable of hemagglutinating human erythrocytes (7,8). With pathogenic E. coli, this property is often associated with a fimbriae that are able to bind to human epithelial cells.

A. hydrophila is currently considered a human pathogen and has been for some time (9,10). More recently, strains of A. hydrophila have been associated with cases of diarrhea in children and adults (4,5,11,12). However, this organism is ubiquitous and is frequently found in water and in the stools of healthy persons (4,6,9,13,14). Although a statistical relationship exists between illness and fecal recovery of A. hydrophila, definite proof of pathogenicity for man is lacking. Oral administration of whole cultures to rhesus monkeys, generally felt to be an animal model closely resembling human infection, failed to cause diarrhea (4). This study was designed to determine

if A. hydrophila strains were capable of causing diarrhea in humans when challenged with whole, viable cultures.

We have conducted a number of studies in Mexico looking at the epidemiology, etiology, therapy and prevention of diarrhea in young adults from the U.S. during short term stay there (1,2,15,16). This setting closely resembles the situation of U.S. military populations during short term relocation in the developing world. We became interested in the observation that antimicrobial agents could successfully treat and prevent the diarrhea even when an agent could not identified in stool (1,2,17). Through a study of serotype and recognized virulence properties of the E. coli isolated from diarrheal stools we identified an agent heretofore not described to be associated with diarrhea (18,19). Initially, we failed to identify common serogroups among the E. coli isolated from diarrheal stools, and furthermore found that enteropathogenic E. coli serogroups were encountered only rarely. Of perhaps greater interest, however, E. coli from illness cases commonly were shown to adhere to HEP-2 cells, a model of pathogenicity for EPEC strains (20). Strains of so called enteroadherent E. coli (EAEC) were found in 42 of 349 (12%) of illness specimens and 7 of 121 (6%) of controls. When looking at the group of students without other agents, 26 of 89 (29%) possessed an EAEC. Thirteen students with an EAEC in diarrheal stool furnished paired sera for serologic study. Six of 13 (46%) showed a fourfold or greater rise in antibody titer to somatic antigen of the isolated E. coli strain (19). The present study was designed to confirm the virulence of EAEC for man and to determine the pathogenesis of infection. These studies offer evidence of a new cause of diarrhea and raise important questions as to the current thinking about relationship of serotype to pathogenicity among E. coli.

Approach to Problem

Aeromonas Studies

Test Strains

Five human isolates of A. hydrophila were selected for virulence assays and volunteer studies (Table 1). All strains were lyophilized upon receipt and stored at room temperature. A new lyophile of the same lot was used for each study.

Biochemical Characterization

All strains were confirmed as A. hydrophila using the API-20E identification system (Analytab Products, Plainview, NY). In addition, strains were plated on DNase test agar (Difco, Detroit, MI) supplemented with oxgall (Difco) and crystal violet (Difco), as previously described (21).

Antimicrobial Susceptibility Testing

Antibiograms were determined using a standard agar diffusion (Kirby-Bauer) method. All strains were tested for susceptibility to ampicillin (AM-10), tetracycline (TE-30), trimethoprim/sulfamethoxazole (SXT), gentamicin (GM-10), furazolidone (FX-100), doxycycline (D-10), and sulfisoxazole (G-.25). Standard ATCC E. coli, S. aureus, S. fecalis and Ps. aeruginosa were used as control strains.

Toxin Assays

Hemolysin activity was assayed using sheep and rabbit blood agar plates (Remel Media, Houston, TX). Strains were inoculated onto both blood agar plates. After an overnight incubation, the plates were examined for beta hemolysis.

Cytotoxin activity was measured in Y-1 adrenal cells (YAC). Cell free supernatants, prepared as previously described (21,22), were added in 100 μ l amounts to confluent YAC monolayers. Following an 18-24 hour incubation, the monolayers were examined for cytotoxic activity (detached cells). Supernatants that caused 100% cytotoxicity were considered positive.

Enterotoxin activity was measured as previously described using the suckling mouse assay (21) and the rabbit ileal loop model (23). Cholera toxin cross-reactive factor (CTCRF) was measured in a ganglioside ELISA (22), using purified cholera toxin to produce a standard curve.

Invasiveness or the ability to invade tissues was assayed in the Sereny eye model (24). Overnight CYE broth cultures (0.1 mL) were inoculated into the eye of adult Hartley strain guinea pigs (Charles River, Wilmington, MA). The animals were examined for keratoconjunctivitis for 8 days. A second method was used to screen for possible invasive capability by the test strains. Overnight CYE broth cultures (1.0 mL) were injected into ligated rabbit ileal loops. The animals were sacrificed after 18 hours and the ligated loops were examined for evidence of invasion and fluid secretion. In both assays, virulent S. sonnei 53GI was used as a positive control.

Hemagglutination Assays

Mannose-sensitive and mannose-resistant hemagglutination patterns were determined using the method of Evans et al. (25). Human (type A), bovine, (Flow Laboratories, McLean, VA), chicken (Flow), monkey (Flow) and guinea pig (Flow) erythrocytes were tested in both the presence and absence of D-mannose (Sigma Chemical Corp., St. Louis, MO).

Selection of Enteroadherent E. coli for Testing in Volunteers

Two strains of EAEC were selected for further study in volunteers, JM 189 and JM 221. They were further evaluated since: they did not produce LT or ST; did not produce keratoconjunctivitis in the guinea pig eye; did not belong to EPEC serogroups; were the sole pathogen isolated from cases of diarrhea; and they each were associated with a fourfold or greater rise in serum antibody to the organism's somatic antigen during the episode of illness.

Volunteer Challenge Studies

Volunteers were identified through advertisement in local university newspapers and The Houston Chronicle (city-wide circulation). The advertisements instructed potential volunteers to call a phone number to learn more about the study from a recruiter. Volunteers were then asked to come to Methodist Hospital to hear details about the study a second time and to be scheduled for medical screening. During the screening process, a medical history form was completed and reviewed by a physician and a chest X-ray, EKG, and blood chemistry profile were performed. Consenting healthy adults were

admitted and confined to the Methodist General Clinical Research Center. A prechallenge admission stool sample was cultured for enteropathogens as described (21).

Volunteers abstained from eating and drinking for the 90 minutes prior to and following oral challenge with test organisms. In a double blind study, groups of three or four volunteers were given 2 grams of NaHCO_3 in 150 mL sterile distilled water as follows: 120 mL of the bicarbonate solution was swallowed and 5 minutes later, the remaining 30 mL of bicarbonate liquid plus the challenge inoculum at predetermined levels were rapidly swallowed. The volunteers were carefully monitored for signs of diarrhea defined as three or more unformed stools within 24 hours or two or more unformed stools in 24 hours with systemic or enteric symptoms. All stools passed were collected and cultured for A. hydrophila or EAEC.

Results

Aeromonas Studies

Biochemical Characterization and Antimicrobial Susceptibility

All five strains were confirmed as A. hydrophila biochemically. The occurrence of several biochemical properties was noted, as potential indicators of virulence (Table 2). All of the strains gave a positive Vogues-Praskauer reaction; only two of the strains produced lysine decarboxylase; all were capable of hydrolyzing esculin and produced DNase. The strains were susceptible to the antimicrobial agents used to treat acute bacterial diarrhea.

Toxin Production

All strains were hemolytic for sheep and rabbit erythrocytes and cytotoxic for YAC (Table 3). Three strains (6Y, B158, and 3647) produced fluid accumulation in the suckling mouse and rabbit ligated ileal loop assays. All five test strains produced a cholera-like toxin cross reactive factor in a ganglioside ELISA.

Hemagglutination Assays

The strains failed to show mannose-resistant hemagglutination with the erythrocytes obtained from five animal species. Strain B158 showed mannose-sensitive agglutination for guinea pig erythrocytes.

Invasiveness

None of the A. hydrophila strains were able to induce keratoconjunctivitis in guinea pigs. All five strains did, however, produce fluid secretion in the rabbit ileal loops along with a purulent hemorrhagic discharge.

Volunteer Challenge Studies

Using the five *Aeromonas* strains, we failed to demonstrate the development of diarrheal illness in 55 of 57 volunteers even with the doses of 10^{10} colony forming units for three of the strains (Table 4). Three strains (B158, SSU and 3284) were not recovered from the stools of the volunteers. Strain 6Y was recovered from 11 of the 20 volunteers challenged. One of the volunteers

developed mild diarrhea (passage of six unformed stools over 12 hours associated with a period of nausea, vomiting, anorexia and malaise) 48 hours after ingesting 3×10^9 colony forming units. He was not treated and failed to develop a progressive enteric illness. A small bowel biopsy obtained shortly after passing the unformed stool was normal histologically. No illness occurred among the four individuals ingesting a higher dose of 6Y (4×10^{10}) casting doubt on the importance of the illness in the one volunteer seen at the lower dose. Strain 3647, earlier identified in a diarrheal stool from a patient in Australia, was recovered from three of the 16 volunteers challenged. One volunteer receiving a dose of 10^7 passed three soft stools over an 18 hour period of time. He had mild abdominal cramps, but did not excrete the test organism. Illness did not occur as the dose was increased and the organism was administered to additional volunteers.

Table 5 summarizes the results obtained when volunteers were fed two dosage levels of the two EAEC strains. When the two strains were ingested in a dose of 7×10^8 cells by the eight volunteers, two of four subjects experienced diarrhea as a result of exposure to strain 221 and one of four developed diarrhea following ingestion of strain 189. Three of four subjects receiving strain 189 in the lower dose experienced malaise, myalgias and abdominal pain. When the dose was increased to 10^{10} cells, two of the four individuals ingesting strain 221 experienced a diarrheal illness with an incubation period of 28 hours, and three of four experienced additional symptoms of enteric infection (malaise, vomiting, abdominal pain). Illness did not develop in the four volunteers receiving the higher dose of strain 189. Sixteen of 16 volunteers fed either strain at the two dosage levels excreted the test strain for three days or longer indicating replication of the strains in the gut.

Discussion and Conclusions

Aeromonas

A. hydrophila, an organism found commonly in the environment, has been statistically associated with diarrheal disease in man in a limited number of parts of the world. Its pathogenicity for selected patients is certain, particularly in skin infections and septicemia. We tested five representative strains of A. hydrophila based on their possession of well characterized virulence properties as well as their association with diarrheal disease. Diarrheal illness failed to occur in 57 volunteers fed varying doses of the characterized strains. The strains did not efficiently colonize the gut of the volunteers as demonstrated by the resultant fecal shedding patterns.

One of the two following conclusions appear to explain the results of the present study:

1. Virulence properties of A. hydrophila as we now understand them - biochemical characteristics and production of hemolysin, cytotoxin, and enterotoxins are insufficient to explain virulence for man.
2. Widespread immunity to A. hydrophila exists among adults from Houston, Texas.

We feel that the latter is not a reasonable explanation for failure to produce illness in view of the rarity of isolating Aeromonas from diarrheal

stools of infants and children from Houston studied by our group over the past 10 years (26,27). Perhaps we are at the point with *Aeromonas*, in terms of understanding its virulence characteristics, where we found ourselves in the early 1970's for *Escherichia coli*. It is possible that the strains of *Aeromonas* we tested, lack the necessary fimbriae to initiate colonization, the first step in pathogenesis. Even though the strains possessed hemagglutinins, which may be associated with fimbriae, these fimbriae may not be intestinal epithelial cells adhesins for *Aeromonas*. Previously recommended biochemical testing (6) was not useful in differentiating strains with virulence for man. Additional virulence properties of *Aeromonas* strains need to be sought and identified before future volunteer studies are likely to be rewarding. Also, we know that enteric bacteria normally pathogenic for only infants (i.e., EPEC) can produce diarrhea in adults when given in the doses employed here (28).

Enteroadherent *E. coli*

Our studies have indicated that *Escherichia coli* which are identified only by their HEp-2 cell adherence property are as commonly associated with acute diarrhea in young adults traveling to Mexico as are strains of shigella. That these strains actually caused the illness in this setting is suggested by a variety of findings: 1. The agents often were the sole agent identified in diarrheal stools; 2. They were isolated in a lower frequency from asymptomatic individuals when compared to illness cases; 3. Humoral antibody development to somatic antigens of the organism commonly occurred during infection; and 4. A limited number of volunteers exposed in Houston to 10^8 - 10^{10} cells experienced clinical illness resembling the disease originally studied in Mexico.

Table 1. Source of Strains of
Aeromonas hydrophila

<u>Strain</u>	<u>Location</u>	<u>Site of Infection</u>
6Y	Bangkok (Echeverria)	Stool (asymptomatic)
B15P	Perth (Gracey)	Wound
3647	Perth (Gracey)	Stool (diarrhea)
SSU	U.S.-C.D.C. (C. Houston)	Stool (diarrhea)
3284	Perth (Gracey)	Stool (diarrhea)

Table 2. Biochemical Characterization and Antimicrobial
Susceptibility of A. hydrophila Strains

Strain	Biochemical Reactions				Antibiogram*						
	Voges Praskauer Reaction	Lysine Decarb. Production	Esculin Hydrolysis	DNase Production	Am	Te	SXT	GM	Fx	DX	G
6Y	+	-	+	+	S	S	S	S	S	S	S
B158	+	-	+	+	R	S	S	S	S	S	S
3647	+	-	+	+	R	S	S	S	S	S	S
SSU	+	+	+	+	S	S	S	S	S	S	S
3284	+	+	+	+	R	S	S	S	S	S	S

*Am - Ampicillin, Te - Tetracycline, SXT - Trimethoprim/sulfamethoxazole, GM - amicin, Fx - Furazolidone, DX - doxycycline, G - Sulfisoxazole.

Table 3. Toxins Produced and Hemagglutination Patterns
of A. hydrophila Strains

train	Toxin Production					Hemagglutination Pattern				
	Hemolysin	Cytotoxin	Suckling Mouse	Rabbit ileal loop	CTCRF	Human	Red Blood Cells Tested			Guinea Pig
							Bovine	Chicken	Monkey	
6Y	+	+	+	+	+	-	-	-	-	-
158	+	+	+	+	+	-	-	-	-	MS*
647	+	+	+	+	+	-	-	-	-	-
SSU	+	+	-	-	+	-	-	-	-	-
284	+	+	-	-	+	-	-	-	-	-

Mannose Sensitive hemagglutination only.

Table 4. Administered Dose and Excretion of

A. hydrophila in Volunteers

Strain	Challenge Dose	Number Volunteers	Number Shedding Test Strain	Diarrhea ⁺
6Y	2x10 ⁴	4	1	0
	1x10 ⁶	4	1	0
	7x10 ⁷	4	4	0
	3x10 ⁹	4	3	1
	4x10 ¹⁰	4	2	0
B158	6x10 ⁴	4	0	0
	2x10 ⁷	4	0	0
3647	1x10 ⁷	4	0	1
	4x10 ⁷	4	0	0
	2x10 ⁹	4	2	0
	3x10 ¹⁰	4	1	0
SSU	4x10 ⁸	4	0	0
	5x10 ⁸	3	0	0
3284	3x10 ⁸	3	0	0
	1x10 ¹⁰	3	0	0

+ - 3 unformed stools/24 hrs or 2 unformed stools/24 hrs with systemic or enteric symptoms.

Table 5. Enteroadherent Escherichia coli Volunteer Studies

<u>Strain (no.)</u>	<u>Dose</u>	Number with <u>Diarrhea (1)</u>	Number with <u>Enteric Symptoms (2)</u>	Number Shedding (3) <u>(range in days)</u>
189 (4)	7×10^8	1/4	3/4	4/4 (1-5)
189 (4)	1×10^{10}	0/4	0/4	4/4 (4)
221 (8)	7×10^8	2/8	1/8	7/7* (3-6)
221 (8)	1×10^{10}	3/8	5/8	8/8 (1-5)

(1) diarrhea = 3 unformed stools/24 hr or 2 unformed stools/24 with an enteric symptom.

(2) enteric symptoms = fever, malaise, vomiting or abdominal pain.

(3) no volunteer shed EAEC prior to ingestion of the organisms.

* one volunteer did not furnish specimen.

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